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Shikonin, a Component of Chinese Herbal Medicine, Inhibits Chemokine Receptor Function and Suppresses Human Immunodeficiency Virus Type 1

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Shikonin is a major component of zicao (purple gromwell, the dried root of *Lithospermum erythrorhizon*), a Chinese herbal medicine with various biological activities, including inhibition of human immunodeficiency virus (HIV) type 1 (HIV-1). G protein-coupled chemokine receptors are used by HIV-1 as coreceptors to enter the host cells. In this study, we assessed the effects of shikonin on chemokine receptor function and HIV-1 replication. The results showed that, at nanomolar concentrations, shikonin inhibited monocyte chemotaxis and calcium flux in response to a variety of CC chemokines (CCL2 [monocyte chemoattractant protein 1], CCL3 [macrophage inflammatory protein 1α], and CCL5 [regulated upon activation, normal T-cell expressed and secreted protein]), the CXC chemokine (CXCL12 [stromal cell-derived factor 1α]), and classic chemoattractants (formylmethionyl-leucine-phenylalanine and complement fraction C5a). Shikonin down-regulated surface expression of CCR5, a primary HIV-1 coreceptor, on macrophages to a greater degree than the other receptors (CCR1, CCR2, CXCR4, and the formyl peptide receptor) did. CCR5 mRNA expression was also down-regulated by the compound. Additionally, shikonin inhibited the replication of a multidrug-resistant strain and pediatric clinical isolates of HIV in human peripheral blood mononuclear cells, with 50% inhibitory concentrations (IC_{50}s) ranging from 96 to 366 nM. Shikonin also effectively inhibited the replication of the HIV Ba-L isolate in monocytes/macrophages, with an IC_{50} of 470 nM. Our results suggest that the anti-HIV and anti-inflammatory activities of shikonin may be related to its interference with chemokine receptor expression and function. Therefore, shikonin, as a naturally occurring, low-molecular-weight pan-chemokine receptor inhibitor, constitutes a basis for the development of novel anti-HIV therapeutic agents.

Zicao (purple gromwell), the dried root of *Lithospermum erythrorhizon* Sieb. et Zucc, *Arnebia euchroma* (Royle) Johnst, or *Arnebia guttata* Bunge, is a commonly used herbal medicine in China and other countries. Earlier studies have demonstrated that zicao extract possesses multiple pharmacological activities, including inhibition of human immunodeficiency virus (HIV) type 1 (HIV-1)-induced cytopathology (27, 32). Shikonin is a major active chemical component isolated from zicao with a molecular weight of 288 (Fig. 1). Like its genitor plant, shikonin possesses numerous pharmacological properties, including anti-inflammatory and antitumor properties and the ability to promote wound healing activity (6, 28).

Chemokine receptors comprise a family of seven transmembrane domain G protein-coupled receptors which play important roles in the immune response, and selected receptors act as coreceptors for HIV-1 (19). The pharmacological profile of zicao led us to study the potential effect of shikonin on chemokine receptor function and on HIV infection. Our previous work showed that shikonin blocks ligand binding to CC chemokine receptor 1 (8). In this report we show that at noncytotoxic concentrations shikonin inhibited leukocyte migration induced by a broad spectrum of chemokines and other chemoattractants. The compound also attenuated chemokine receptor-mediated calcium flux and induced a decrease in human monocyte/macrophage surface expression of CCR1, CCR2, CXCR4, and the formyl peptide receptor (FPR). However, the decrease was more pronounced in the case of CCR5, and the level of expression of CCR5 mRNA in human monocytes/macrophages was also decreased by shikonin. Furthermore, shikonin inhibited HIV-1 replication in primary human peripheral blood mononuclear cells (PBMCs) and macrophages. These results suggest that shikonin is a pan-chemokine inhibitor that blocks chemokine receptor function by interfering with the downstream signal, and this property may account for its ability to suppress inflammation and HIV-1 replication.

MATERIALS AND METHODS

Reagents and cells. Shikonin (Fig. 1) was obtained from TCI (Tokyo, Japan). Human blood enriched with mononuclear cells was obtained from healthy donors by leukapheresis (Transfusion Medicine Department, Clinical Center, National Institutes of Health, Bethesda, Md.) with the approval of the donors according to the human subjects research policies of the National Institutes of Health. PBMCs collected at the interface were washed with phosphate-buffered saline (PBS) and centrifuged through a 46% isosmotic Percoll gradient (Pharmacia, Uppsala, Sweden). Monocytes were further purified (purity, >90%). The

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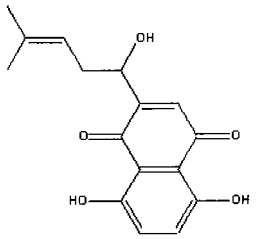

FIG. 1. Chemical structure of shikonin ($C_{16}H_{16}O_5$; molecular weight, 288.2994).

cells were resuspended in RPMI 1640 medium containing 10% fetal calf serum (HyClone, Logan, Utah) for further use. The Ba-L isolate of HIV was obtained from the AIDS Research and Reference Reagent Program, National Institutes of Health. Pediatric clinical isolates Wejo, Teki, Rojo, and SLKA were derived as described previously (4). Multidrug-resistant HIV isolate MDR-769 was obtained from Tom Merrigan, Stanford University, La Jolla, Calif. It was derived from a highly antiretroviral agent-experienced patient expressing resistance to the reverse transcriptase and protease inhibitors 3′-azido-3′-deoxythymidine (AZT), dideoxyinosine, stavudine, lamivudine, nevirapine, foscarnet, saquinavir, indinavir, and nelfinavir.

Cell viability. Freshly isolated human monocytes were plated in a 96-microwell plate (Costar) at 10^5 cells per well with shikonin at a final concentration of 10^{-5}, 10^{-6}, 10^{-7}, or 10^{-8} M. The numbers of viable cells, as detected by the standard 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS), test (16) and the trypan blue exclusion assay, were measured 12 and 24 h after incubation at 37°C in humidified air with 5% CO_2. The effect of shikonin on cell viability was expressed as percent viability (percentage of control group viability), which was calculated by the equation (EG × 100)/CG, where CG and EG are the optical densities at 490 nm for the control group and the experimental group, respectively.

Chemotaxis assay. Leukocyte migration was assessed by a 48-well microchemotaxis chamber technique, as described previously (12). Stimulants were diluted in chemotaxis medium (RPMI 1640 medium, 1% bovine serum albumin, 25 mM HEPES) and placed in the lower wells, while 50 μl of cell suspension pretreated with different concentrations of shikonin or with the vehicle (10^6 cells/ml in chemotaxis medium) was placed in the upper wells (Neuroprobe, Cabin John, Md.). The two compartments were separated by a polycarbonate filter (pore size, 5 μm; Neuroprobe). After incubation at 37°C in humidified air with 5% CO_2 (90 min for monocytes, 60 min for neutrophils), the filter was removed, fixed, and stained with Diff-Quik (Harlew, Gibbstown, N.J.). The filters were analyzed with the BIOQUANT imagine analysis system (R & M Biometrics, Nashville, Tenn.) at ×200 magnification. The data were expressed as the chemotactic index, which represents the fold increase in the number of cells migrating in response to chemoattractants over the cell response to the control medium. The percent inhibition of chemotaxis was calculated by the following formula: $[1 - (EG/CG)] \times 100$, where EG is the migratory cell number for the experimental group minus the number of spontaneously migrating cells, and CG is the migratory cell number for the chemokine- and chemoattractant-positive control groups minus the number of spontaneously migrating cells.

Calcium mobilization. Cells were assayed for intracellular calcium mobilization as described previously (7). Human monocytes resuspended at 10^7 cells/ml in loading buffer (138 mM NaCl, 6 mM KCl, 1 mM $CaCl_2$, 10 mM HEPES, 5 mM glucose, 0.1% bovine serum albumin [pH 7.4]) were incubated with 5 μM Fura-2 dye (Sigma) at 37°C for 30 min. The dye-loaded cells were washed twice with Dulbecco's modified Eagle's medium with 10% fetal bovine serum and once with loading buffer and were then resuspended in fresh loading buffer at a concentration of 5×10^5cells/ml. Two milliliters of cell suspension was pipetted into quartz cuvettes in a luminescence spectrometer (LS50 B; Perkin-Elmer Limited). Stimulants (volume, 20 μl) were added to the cuvettes at the indicated time points. The ratio of the fluorescence at a wavelength of 340 to that at a wavelength of 380 nm was calculated by using the FL WinLab program (Perkin-Elmer).

Flow cytometry. The effects of shikonin on CCR1, CCR2, CCR5, CXCR4, and FPR surface expression on monocytes/macrophages were evaluated through the courtesy of L. Finch, SAIC Frederick, Inc., National Cancer Institute—Frederick. Human monocytes in medium supplemented with 20 ng of monocyte colony-stimulating factor (M-CSF) per ml were incubated for 24 h. Shikonin (1 μM) was added to the culture medium, and the mixture was cultured for another 24 h. Monocytes/macrophages were detached by gently scraping them in ice-cold PBS without calcium and magnesium and were then washed with PBS. They were then stained with control monoclonal antibody (MAb; MAb MsIgG; Coulter Clone) or purified anti-human CCR1 (CCR1-111; RDI, Flanders, N.J.), CCR2 (48607; R&D), or FPR (5F1, PharMingen) MAb, followed by staining with goat anti-mouse immunoglobulin G (IgG; Fc specific)–fluorescein isothiocyanate (FITC) antibody (Sigma), or were stained with FITC-conjugated isotype-matched mouse IgG (PharMingen), FITC-conjugated anti-human CCR5 MAb (MAb 2D7; PharMingen), or anti-human CXCR4 MAb (MAb 12G5; R&D). In another set of experiments, M-CSF-differentiated human monocytes/macrophages were treated with 0.1 to 50 μM shikonin at 37 or 4°C for 2 h and were then subjected to CCR5 expression analysis. HEK/CCR5 cells were detached from the tissue culture flask by using trypsin-free medium, the cell suspension was treated with the desired concentration of shikonin, and the mixture was incubated for 60 min at 37°C. Under the control conditions, the cells were incubated for 20 min with or without RANTES (regulated upon activation, normal T-cell expressed and secreted protein; 100 ng/ml). After the cells were washed with ice-cold PBS, they were treated with fixing buffer for 5 min on ice, followed by another washing with ice-cold PBS. The cells were then stained with FITC-conjugated isotype-matched mouse IgG or FITC-conjugated isotype-matched anti-CCR5 MAb. Stained cells were analyzed on an EPICS profiler (Coulter Corp., Miami, Fla.). The data are expressed as the mean fluorescence intensity and the percentage of positive cells.

RNA isolation and RT-PCR. Total RNA from monocytes/macrophages differentiated with M-CSF for 48 h was isolated by use of the TRIzol reagent (Life Technologies, Grand Island, N.Y.). Shikonin was added to the cell suspension at the last 24-h incubation period. Reverse transcriptase (RT) PCR (RT-PCR) was performed with the GeneAmp RNA PCR kit (Roche Molecular Systems, Branchburg, N.J.). Thirty cycles were used for the amplification of CCR5 and β-actin sequences. The primers specific for CCR5 were 5′-TAACAGGTTGGA CCAAGCTAT-3′ and 5′-CACTTGAGTCCGTGTCACA-3′. PCR products were identified on 2% agarose gels after ethidium bromide staining and were documented photographically. To ensure the quality of the procedure, RT-PCR was also performed with primers specific for β-actin. The relative levels of expression of CCR5 were quantified with the ImageJ program.

Anti-HIV-1 assays with human PBMCs and monocytes/macrophages. Human PBMCs and monocytes were isolated from hepatitis virus- and HIV-seronegative donors by Ficoll-Hypaque gradient centrifugation. Assays for anti-HIV-1 activity were accomplished with 3-day-old phytohemagglutinin- and interleukin-2-stimulated PBMCs or 6-day-old cultured monocytes/macrophages. All experiments for antiviral activity were performed with triplicate samples in RPMI 1640 medium supplemented with 15% fetal bovine serum, L-glutamate (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). HIV-1 replication in PBMC and monocyte/macrophage cultures was determined by measurement of the RT activity in the supernatant and p24 antigen expression, respectively, by enzyme-linked immunosorbent assay (Coulter, Hialeah, Fla.) (2, 3). Cell viability was determined by measurement of formazan dye reduction in replicate cultures in CellTiter reagent (Promega, Madison, Wis.). The HIV RT inhibitor AZT was used as a positive control for all assays.

Statistical analysis. All experiments were performed at least three times, and the results of representative experiments are presented. All data are expressed as means ± standard errors of the means. The significance of the difference between the experimental and control groups was analyzed by a Student's *t* test.

RESULTS

Effects of shikonin on human monocyte and PBMC viability. The MTS assay showed that no toxic effects were observed after coincubation of shikonin over a concentration range of 1 nM to 1 μM with human monocytes for up to 24 h. This observation was confirmed by the trypan blue exclusion assay (Fig. 2). The cytotoxic effects of shikonin on human PBMCs and monocytes/macrophages were measured in parallel by an HIV infection assay, as described below.

Shikonin inhibits leukocyte migration induced by chemoattractants. As shown in Fig. 3A, freshly isolated human monocytes migrated to complement fraction C5a (10^{-9} M), CCL5 (RANTES; 100 ng/ml), CCL2 (monocyte chemoattractant protein 1; 100 ng/ml), CXCL12 (stromal cell-derived factor 1α;

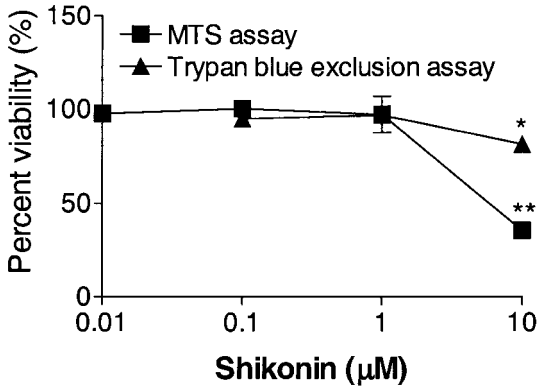

FIG. 2. Effect of shikonin on human monocyte viability. Freshly isolated human monocytes were placed in 96-well plates at 10^5 cells/well (200 μl) with the indicated concentrations of shikonin. After incubation for 24 h at 37°C in humidified air with 5% CO_2, cell viability was measured by the standard MTS assay and the trypan blue exclusion assay. Data are expressed as the means ± standard errors of the means for quadruplicate samples. *, $P < 0.05$ compared with the results for the control group (medium only); **, $P < 0.01$ compared with the results for the control group (medium only).

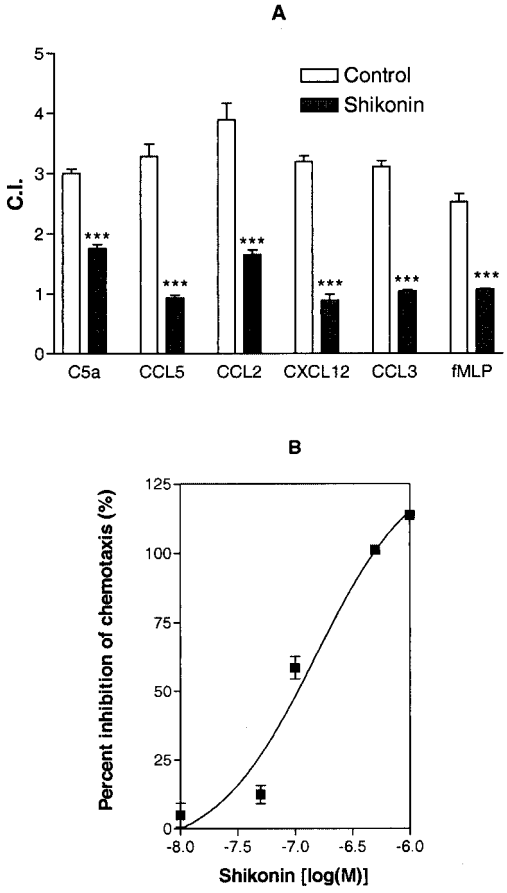

FIG. 3. Effect of shikonin on chemokine- and chemoattractant-induced human monocyte chemotaxis. (A) Chemotactic response of monocytes (treated with 0.5 μM shikonin) to C5a (10^{-9} M), CCL5 (RANTES; 100 ng/ml), CCL2 (monocyte chemoattractant protein 1; 100 ng/ml), CXCL12 (stromal cell-derived factor 1α; 100 ng/ml), CCL3 (macrophage inflammatory protein 1α; 100 ng/ml), and formylmethionyl-leucine-phenylalanine (fMLP; 10^{-9} M); (B) shikonin-inhibited monocyte migration induced by CCL5 (RANTES; 100 ng/ml) in a dose-dependent manner. ***, $P < 0.001$ compared with the results for the control group (chemokine or chemoattractant only). C.I., chemotactic index.

100 ng/ml), CCL3 (macrophage inflammatory protein 1α; 100 ng/ml), and formylmethionyl-leucine-phenylalanine (10^{-8} M). In the presence of 0.5 μM shikonin, the chemotactic responses of monocytes to chemoattractants were significantly ($P < 0.001$) inhibited. Shikonin exhibited a dose-dependent inhibition of human monocyte migration induced by CCL5 (50% inhibitory concentration [IC_{50}], < 0.1 μM) (Fig. 3B). In another set of experiments, human monocytes were pretreated with 0.5 μM shikonin for 15 min at room temperature, followed by thorough washing, before testing for chemotaxis. The migratory responses of these cells to chemoattractants were also markedly inhibited ($P < 0.001$) (data not shown).

Shikonin attenuates leukocyte calcium flux. Human leukocytes respond to stimulation with chemoattractants with a transient mobilization of intracellular calcium. In human monocytes, calcium mobilization was evoked by CCL5 (100 ng/ml), CCL2 (100 ng/ml), and formylmethionyl-leucine-phenylalanine (10^{-9} M) (Fig. 4A). Treatment of monocytes with 1 μM shikonin resulted in a time-dependent attenuation of calcium mobilization. As shown in Fig. 4B to E, addition of shikonin 30 s before stimulation with chemoattractants had no significant effect on calcium mobilization. However, pretreatment for 10 min markedly suppressed calcium mobilization. Calcium mobilization was completely inhibited following 30 min of coincubation of the cells with shikonin.

Shikonin down-regulates chemokine receptor expression on human monocytes/macrophages. The expression of CCR5 on the surfaces of freshly isolated human monocytes was virtually undetectable by flow cytometry with MAb 2D7 (data not shown). Culture in the presence of M-CSF for 48 h increased the proportion of cells expressing CCR5 up to 63.9% (Fig. 5, CCR5 control), consistent with previously published data (29). The addition of shikonin (1 μM) for the last 24 h during the 48-h culture period decreased the level of CCR5 surface expression by 80% (Fig. 5, CCR5 with shikonin treatment). To a lesser extent, the surface expression of CXCR4, CCR1, CCR2, and FPR was also down-regulated by 24 h of treatment with shikonin (Fig. 5), suggesting that CCR5 is more sensitive to shikonin. The down-regulation of CCR5 surface expression on monocytes/macrophages by treatment with shikonin was observed as early as 2 h after treatment. At 37°C, the IC_{50} of shikonin for the inhibition of CCR5 expression was less than 1 μM. However, at 4°C, the inhibitory effect of shikonin on CCR5 surface expression was substantially reduced (Fig. 6).

Shikonin down-regulates CCR5 mRNA expression in human monocytes/macrophages. The CCR5 receptor transcripts in monocytes were virtually absent until differentiation (20). Consistent with previous reports, our observation showed that after 48 h of stimulation with M-CSF, CCR5 mRNA became detectible in human monocytes by RT-PCR. Treatment with 1 μM shikonin for 24 h resulted in a marked decrease in the level of CCR5 transcription (Fig. 7A and B).

Shikonin inhibits HIV-1 replication. We then evaluated the effect of shikonin on HIV-1 replication in PBMCs and monocytes/macrophages. Figure 8 shows that shikonin inhibited the

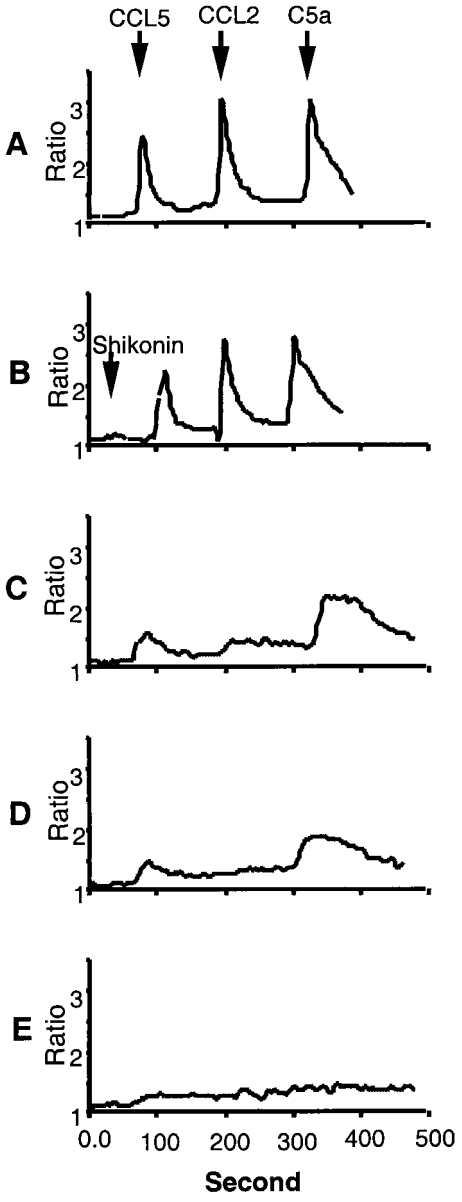

FIG. 4. Effect of shikonin on calcium mobilization evoked by chemoattractants. Human monocytes were loaded with a dye (Fura-2) and then stimulated with CCL5 (RANTES; 100 ng/ml), CCL2 (monocyte chemoattractant protein 1; 100 ng/ml), and C5a (10^{-9} M), as indicated. The ratio of the fluorescence at a wavelength of 340 to the fluorescence at a wavelength of 380 nm was recorded and calculated by using the FL WinLab program. (A) Calcium mobilization induced by CCL5 (RANTES), CCL2 (monocyte chemoattractant protein 1), and C5a. Shikonin (1 μM) was then added to the cell suspension for 50 s (B), 10 min (C), 20 min (D), and 30 min (E) before stimulation with chemoattractants.

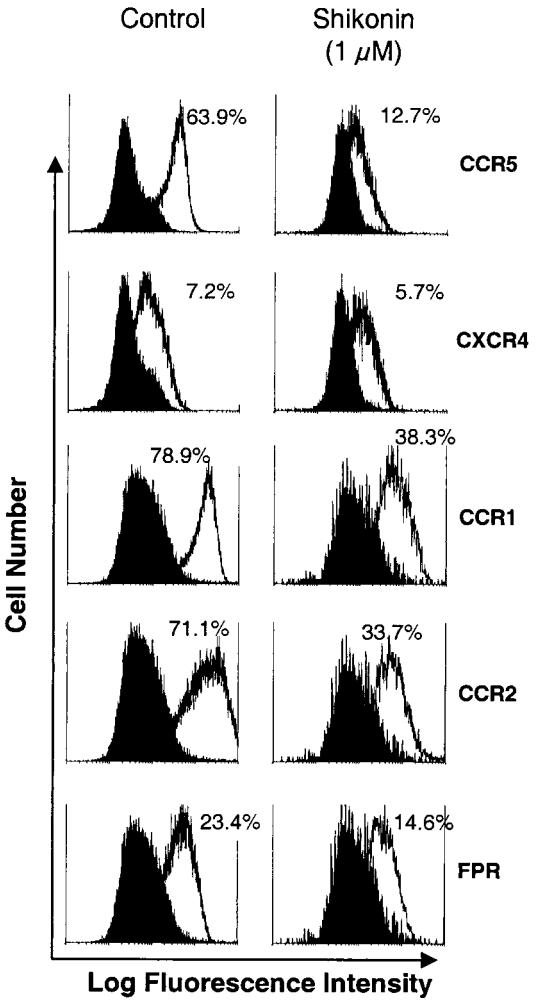

FIG. 5. Effects of shikonin on chemokine receptor expression on human monocytes/macrophages. Human monocytes in medium supplemented with 20 ng of M-CSF per ml were incubated for 24 h. Shikonin (1 μM) was added to the medium, and the monocytes were incubated for another 24 h. The cells were detached, washed with PBS, and then stained with control MAb or purified anti-human CCR1, CCR2, or FPR MAb, followed by staining with goat anti-mouse IgG (Fc specific)–FITC antibody, or were stained with FITC-conjugated isotype mouse IgG, FITC-conjugated anti-human CCR5 MAb, or anti-human CXCR4 MAb. Stained cells were analyzed on and EPICS profiler (Coulter Corp.). Data are expressed as the percentage of positive cells. The black histograms show the results for the isotype-matched controls.

replication of clinical HIV isolates RoJo, Wejo, Teki, and SLKA in PBMCs with IC_{50}s of 126, 366, 96, and 344 nM, respectively (Fig. 8A to D). Shikonin also inhibited the replication in PBMCs of multidrug-resistant HIV isolate MDR-769 (resistant to AZT, dideoxyinosine, stavudine, lamivudine, nevirapine, foscarnet, saquinavir, indinavir, and nelfinavir), with an IC_{50} of 252 nM (Fig. 8E). HIV Ba-L replication in monocytes/macrophages was also inhibited by the compound, with an IC_{50} of 470 nM (Fig. 8F). All assays were performed with either AZT or dextran sulfate (with isolate MDR-769 only) as positive controls and resulted in IC_{50}s of between 2 and 5 nM for AZT and 2.2 μg/ml for dextran sulfate. The effects of shikonin on human PBMCs and monocytes were measured in parallel by an HIV infection assay. Meanwhile, the 50% cytotoxic concentrations of shikonin for PBMCs and monocytes/macrophages were 1.9 and 47.3 μM, respectively. The increased cytotoxic effect of shikonin on PBMCs was supported by observations that shikonin inhibited lymphocyte proliferation (data not shown). The macrophages appeared to be more resistant to shikonin-mediated cell death than the freshly isolated monocytes (Fig. 2), which is consistent with the findings

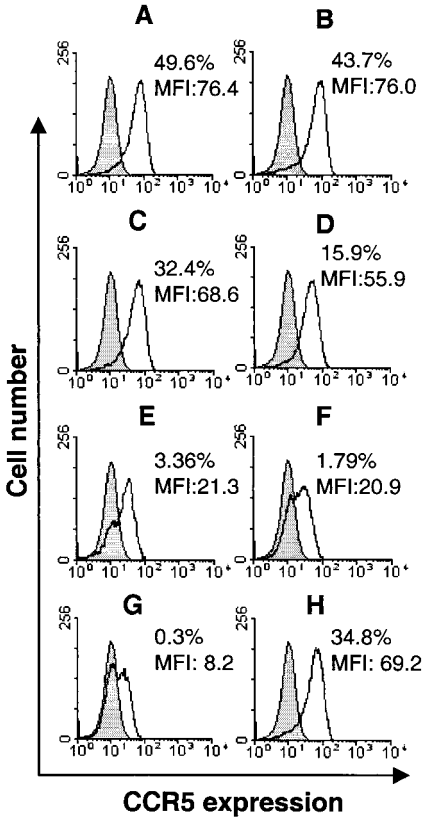

FIG. 6. Dose-dependent inhibition of CCR5 surface expression on human monocytes/macrophages by shikonin. Human monocytes were incubated for 46 h in medium supplemented with 20 ng of M-CSF per ml. Shikonin was added to the medium at the desired concentration, and incubation was continued for another 2 h at 37 or 4°C. The cells were detached and washed with PBS and then stained with FITC-conjugated isotype-matched mouse IgG or FITC-conjugated anti-human CCR5 MAb. Stained cells were analyzed on an EPICS profiler (Coulter Corp.). (A) Medium control; (B to G) treatment with increasing concentrations of shikonin (0.1, 0.5, 1, 5, 10, and 50 μM, respectively) at 37°C; (H) treatment with 10 μM shikonin at 4°C. Grey histograms, results for the isotype-matched IgG control; open histograms, results with CCR5 antibody staining. MFI, mean fluorescence intensity.

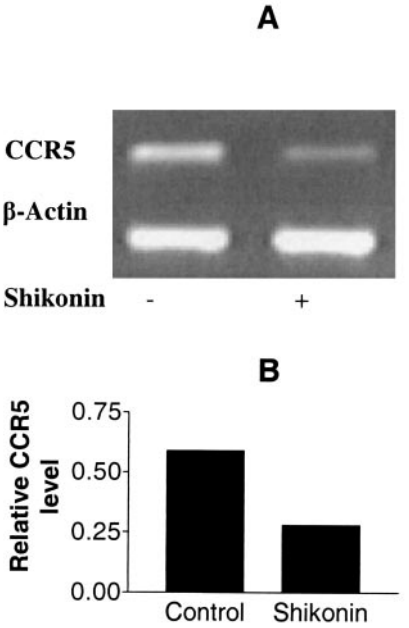

FIG. 7. Down-regulation of CCR5 mRNA expression by shikonin. (A) Human monocytes in medium supplemented with 20 ng of M-CSF per ml were incubated for 24 h. Shikonin (1 μM) was added to the medium, and the monocytes were incubated for another 24 h. The cells were detached and washed with PBS. Total cellular RNA was subjected to RT-PCR analysis, as described in Materials and Methods. (B) The relative mRNA levels represent the ratio of the signal intensity from densitometric readings after normalization with β-actin.

presented in a previous report (21). Thus, shikonin inhibits HIV replication in both monocytes and PBMCs at doses significantly below those required to induce cytotoxic effects.

DISCUSSION

Prompted by the reported anti-inflammatory and anti-HIV-1 activities of its parental herbal extract, we evaluated the effect of shikonin on chemokine receptor function. We initially showed that CCR1 ligand binding to monocytes was selectively blocked by shikonin (8). In this study we focused on monocyte migration induced by a variety of proinflammatory chemoattractants. Our results revealed that shikonin inhibited the human monocyte chemotactic response to a broad spectrum of chemoattractants. Furthermore, we observed that the inhibitory effect of shikonin was not removed by washing shikonin from the cell suspension, suggesting that shikonin interacted irreversibly with cell components that are critical for the function of chemokine receptors. As supporting evidence, the calcium mobilization evoked by chemoattractants was attenuated after 30 min of treatment of cells with shikonin. Shikonin itself did not mobilize calcium in monocytes, indicating that it is not a G protein-coupled receptor agonist but an antagonist.

Although shikonin has been shown to selectively block the CCR1 function in monocytes (8), the surface expression of CCR5 on macrophages and HEK/CCR5 cells (data not shown) was also decreased in a dose-dependent manner. In contrast, treatment of cells with shikonin on ice resulted in much lower level of reduction of CCR5 surface expression, suggesting that the effect of shikonin is dependent on normal cellular metabolism and is not based on its direct interaction with the extracellular domain of CCR5. Treatment of human monocytes/macrophages with shikonin for 24 h resulted in decreased levels of surface expression of all chemoattractant receptors for which tests were conducted, with a more pronounced reduction in CCR5 expression. The down-regulation of chemokine receptor surface expression by shikonin is independent of an increase in the levels of chemokine ligands in the cell culture medium (data not shown). Therefore, interference with chemokine receptor signaling and receptor gene transcription is a plausible mechanism for the effects of shikonin. Shikonin interferes with multiple intracellular molecular events (6), including inhibition of chemoattractant-induced inositol phosphate generation (30). As the G protein plays an initial role in chemokine receptor signal transduction, we hypothesize that shikonin may interfere with G-protein activity and therefore suppresses the downstream signal. However, further study is needed to verify this hypothesis.

Chemokine ligands bind to CCR5 at the second extracellular

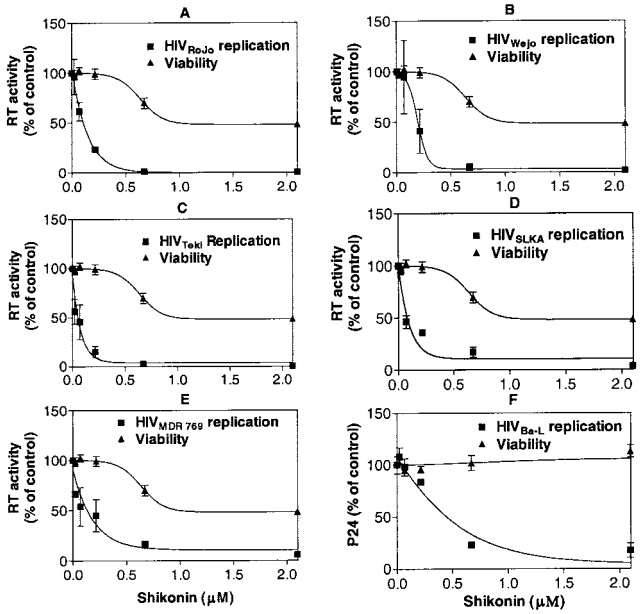

FIG. 8. Suppression of HIV-1 replication in human PBMCs or human macrophages. (A to E) For the RT activity assay, human PBMCs were coincubated with HIV-1 RoJo, Wejo, Teki, SLKA, or MDR-769 for 7 days. HIV-1 RT activity was monitored in vitro by standard RT assays of cell culture supernatants. (F) For p24, macrophages (10^6 cells/well) cultured for 7 days in 24-well plates were incubated with or without shikonin for 2 h before infection with HIV-1_{Ba-L}. The cells were then washed to remove unbound virus. Fresh medium containing shikonin was added. The culture medium was replaced twice weekly. Seven days after infection, HIV-1 p24 core antigen levels were measured by enzyme-linked immunosorbent assay. The cell viability was evaluated simultaneously by MTS assays. The data show the percent activity compared to that for the control.

loop (24), and the CCR5 MAb (31) used in this study, MAb 2D7, also binds to the same receptor domain. MAb 2D7 has been shown to inhibit CCR5 ligand binding to T cells and to induce T-cell chemotaxis, but it failed to inhibit CCR5 ligand binding to monocytes or to induce monocyte chemotaxis (31). Our observation showed that shikonin did not block radiolabeled chemokine ligand binding to the HEK/CCR5 cell line (8) but decreased the level of MAb 2D7 staining of the cells. Of interest is the contrast of shikonin with another low-molecular-weight CCR5 antagonist, TAK-779, which inhibits radiolabeled RANTES binding to CHO-CCR5 cells but which did not inhibit the binding of MAb 2D7 to the cells (1). These observations suggest that a low-molecular-weight antagonist of chemokine receptors may not necessarily block chemokine binding to the cell. For instance, UCB35625 selectively inhibits the CCR1- and CCR3-mediated functions (chemokine-induced shape change and chemotaxis) at nanomolar concentrations but fails to competitively inhibit ligand binding to the receptors (23). In contrast to these receptor-specific antagonists, shikonin appears to interact more broadly with cellular components that are involved in the expression and function of multiple receptors. In addition, at 4°C, shikonin did not reduce the level of CCR5 MAb binding to macrophages, indicating that shikonin does not cause steric hindrance of antibody binding to the receptor.

Coreceptor levels on cells are a critical factor for entry and subsequent infection by HIV-1. Hence, patients with reduced or nonexistent levels of CCR5 on peripheral blood mononuclear cells are resistant to HIV-1 infection (17). In addition, high-level expression of CCL3 (macrophage inflammatory protein 1α) and CCL4 (macrophage inflammatory protein 1β) in the blood provides protection against HIV-1 infection (10). It is possible that CCR5 ligands are able to induce CCR5 internalization, thus reducing the amount of HIV-1 that enters the cell. Shikonin did not inhibit RANTES-induced CCR5 internalization. Furthermore, treatment with both shikonin and CCL5 had synergistic effects on the reduction of CCR5 surface expression (data not shown), which may also contribute to the anti-HIV-1 replication activity of shikonin. Increasing evidence shows that HIV proteins, such as gp120, are capable of interacting directly with chemokine coreceptors, triggering cellular signaling events that may play an important role in the process of infection (26). Thus, the inhibitory effects of shikonin on chemokine receptor signaling may also contribute to its anti-HIV-1 activity. A recent report revealed that the IC_{50} of shikonin for inhibition of the RNase H activity of HIV-1 RT was more than 100 μM (18), reinforcing the notion that the target of shikonin may not be the virus itself but host cell-related activities, such as coreceptor expression and function and signaling transduction. From our observations, shikonin inhibited HIV-1 RoJo (X4 tropic; IC_{50}, 126 nM), Wejo (R5 tropic; IC_{50}, 366 nM), Teki (R5 tropic; IC_{50}, 96 nM), SLKA (X4 tropic; IC_{50}, 344 nM), MDR-769 (X4 tropic; IC_{50}, 252 nM), and Ba-L (R5 tropic; IC_{50}, 470 nM) replication. Our observations suggest that shikonin may be broadly efficient against many HIV-1 strains. However, as shikonin has been shown to have a wide range of biological activities, further studies should be done to verify whether its mechanism of action against HIV-1 results solely from its down-regulation of coreceptor function or from its various biological activities, including direct inhibition of HIV-1 long terminal repeat-directed gene expression or post-transcriptional events.

The use of highly active antiretroviral therapy with RT and protease inhibitors has resulted in the potent and relatively sustained suppression of viral loads in HIV-1-infected individuals (5). However, the discovery of novel anti-HIV-1 agents with diverse mechanisms of action due to the toxic effects of the therapeutic regimen and the emergence of drug-resistant viral variants is essential. In this context, coreceptors involved in HIV-1 entry are receiving special attention as targets (13). CCR5 has been shown to act as a major coreceptor for fusion and entry of macrophage (R5)-tropic HIV-1 (11). R5 strains are prevalent during the asymptomatic stages of HIV-1 infection, whereas T-cell-line (X4)-tropic strains become prevalent only in the symptomatic stages, concomitant with the decline in the levels of $CD4^+$ T cells (14). A 32-bp deletion in the CCR5-coding region (CCR5 Δ32) generates a nonfunctional receptor, and CCR5 Δ32 homozygous individuals are resistant to infection with R5-tropic HIV-1 (17). Natural ligands for CCR5 (RANTES [CCL5], macrophage inflammatory protein 1α [CCL3], macrophage inflammatory protein 1β [CCL4]) and their analogues (Met-RANTES and amino-oxypentane-RANTES) are known to block R5-tropic HIV-1 infection (9, 22, 25). Our studies show that shikonin inhibits both X4-tropic virus and R5-tropic virus, suggesting that shikonin is a potentially useful inhibitor of different HIV-1 strains.

Chemokine-mediated cell migration is the first-line host defense, but leukocyte accumulation and activation also cause tissue damage, which culminates in inflammation, autoimmunity, and graft rejection. Chemokine antagonists with low molecular weights and high levels of bioavailability may have much higher therapeutic potential than steroids in certain disease states, with minimal side effects (15). Shikonin has long been used as an anti-inflammatory agent in traditional medicine (6), and its beneficial activity may be related to its antagonistic action on chemoattractant receptors.

Taken together, shikonin is a naturally occurring low-molecular-weight pan-chemokine receptor antagonist. It inhibited chemokine receptor-mediated leukocyte migration as well as calcium mobilization and down-regulated the levels of surface expression of CCR5 and other chemoattractant receptors. Additionally, shikonin was shown to be an inhibitor of HIV-1 replication, which may be attributed in part to its inhibition of HIV coreceptors. Thus, shikonin may be considered a useful lead compound for the future design and development of anti-HIV-1 and anti-inflammatory therapeutic agents.

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